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14. ABSTRACT

Wnt/beta-Catenin signaling and associated target genes are implicated in the establishment of bone metastasis and in the development of castration resistant prostate cancer. Our previous studies have shown that Foxa2 is a Wnt/beta-catenin target gene in prostate. Our preliminary study suggest a Wnt–Foxa2–CXCR4 axis that is involved in PCa bone metastasis, and activation of this axis provides survival mechanisms for PCa cells following androgen deprivation. The hypothesis is that the Wnt/beta-catenin activation of Foxa2 and CXCR4 promotes progression to CRPCa and facilitates bone colonization by PCa cells, and that targeting this axis will provide a novel treatment for PCa bone metastasis and relapse after androgen ablation. In the past one year, our effort mainly focused on addressing if active Wnt/beta-Catenin signaling induces Foxa and CXCR4 to promote androgen independent prostate cancer cell growth; if knocking down Foxa2 in prostate cancer cells impairs their growth in the bone; and to characterize 22Rv1's growth in the bones.

15. SUBJECT TERMS
Wnt beta-Catenin, Foxa2, CXCR4, prostate cancer, metastasis, castrate resistant

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Introduction

Disease-specific mortality in men with prostate cancer (PCa) is almost exclusively the result of the development of castrate resistant (CR) PCa subsequent to hormone ablation. [1] Hormone ablation is the gold standard treatment for metastatic PCa. However, metastasis is the main contributor to mortality in men with advanced PCa. [2]There is no cure for hormone refractory metastatic PCa. Understanding the mechanisms by which PCa cells form metastasis in bone and progress to CRPCa is critical for the development of novel therapeutics. Wnt/β-Catenin signaling and associated target genes are implicated in the establishment of bone metastasis and in the development of CRPCa. High levels of Wnt-1 expression and its down steam mediator, nuclear β-Catenin, are detected in 85% of PCa bone metastasis. We have reported activation of Wnt/beta-Catenin causes HGPIN and enables the prostate to continuously grow following castration,[3, 4] implicating this pathway in the development of castrate resistance. Our studies have also shown that Foxa2, a forkhead transcription factor, is a Wnt/beta-Catenin target gene in the prostate, and that Foxa2 expression is localized at the invasive front in prostate tumors. Our preliminary study confirmed that Foxa2 is expressed in a subset of human PCa bone metastases, implicating Foxa2 in human PCa bone metastasis. My preliminary data indicate that Foxa2 and beta-catenin directly regulate CXCR4 expression, thus establishing the Wnt–Foxa2–CXCR4 axis. We have evidence supporting that the Wnt-Foxa2-CXCR4 axis is involved in PCa bone metastasis, and activation of this axis provides survival mechanisms for PCa cells following androgen deprivation. The hypothesis is that the Wnt/beta-catenin activation of Foxa2 and CXCR4 promotes progression to CRPCa and facilitates bone colonization by PCa cells, and that targeting this axis will provide a novel treatment for PCa bone metastasis and relapse after androgen ablation. This hypothesis will be tested by the following Specific Aims:

Aim 1: To determine if Wnt/β-Catenin signaling induces Foxa2 and CXCR4 to promote CRPCa growth.

Aim 2: To determine if the expression of Foxa2 facilitates castration resistant PCa growth in the bone.

Aim 3: To determine the suitability of pharmacological inhibition of Wnt-Foxa2-CXCR4 axis in conjunction with hormone deprivation to inhibit PCa growth and CR relapse in the bone.

This research will establish the functional implication of the Wnt-Foxa2-CXCR4 axis in PCa progression (metastasis to bone and CR growth). This study will also determine the suitability of this axis as a novel therapeutic target for treating PCa metastasis and relapse after hormone deprivation.

Body

In the past one year, our effort mainly focused on addressing 1) if active Wnt/beta-Catenin signaling induces Foxa2 and CXCR4 to promote androgen independent prostate cancer cell growth *in vitro***.** (Aim 1a); 2) if knocking down Foxa2 in prostate cancer cells impairs their growth in the bone (aim 2c); and 3) to characterize 22Rv1, a prostate cancer cell line, its' growth in the bones (to optimize cell line and condition for aim 3 study). We conducted the following research as listed in the statement of work:

Task 1: To determine if Wnt/beta-Catenin signaling induces Foxa2 and CXCR4 to promote castration resistant prostate cancer growth.

1a. To determine if active Wnt/beta-Catenin signaling induces Foxa2 and CXCR4 to promote androgen independent prostate cancer cell growth *in vitro***.**

A, We conducted *in vitro* co-culture experiments and found that conditioned medium collected from CAFs activate Wnt/beta-Catenin signaling in epithelial cells. Top-Flash (Wnt reporter) was transfected into prostate epithelial cells and treated with conditioned medium collected from CAFs or NPFs. While conditioned medium collected from NPFs only gave a base line level of luciferase activity, conditioned medium collected from CAFs activate Wnt/beta-Catenin reporter.

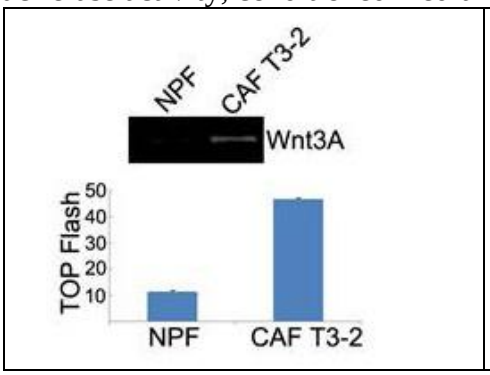


Figure 1. CAF cells secreted Wnt3a and activated Wnt/beta-Catenin pathway in prostate epithelial cells.
Upper panel: RT-PCR to examine the level of canonical Wnt3a expression in NPF and CAF cells.
Lower panel: Wnt-responsive promoter (Top) driven luciferase assay to show that conditioned medium collected from CAF cell culture activated Wnt reporter.

B, We established Foxa2 over-expressing prostate epithelial cell line. Foxa2 was stably expressed in prostate epithelial cell NeoTag1 cells. These cells will be used in later experiments in aim 2.

C, We established Foxa2 knock-down PC3 cells. Foxa2 level was knocked down by shRNA approach in prostate cancer PC3 cells.

D, We conducted proliferation assays and found that Foxa2 over-expression in NeoTag1 cells empowers androgen independent growth; however, knocking down Foxa2 in PC3 cells did not affect cell proliferation.

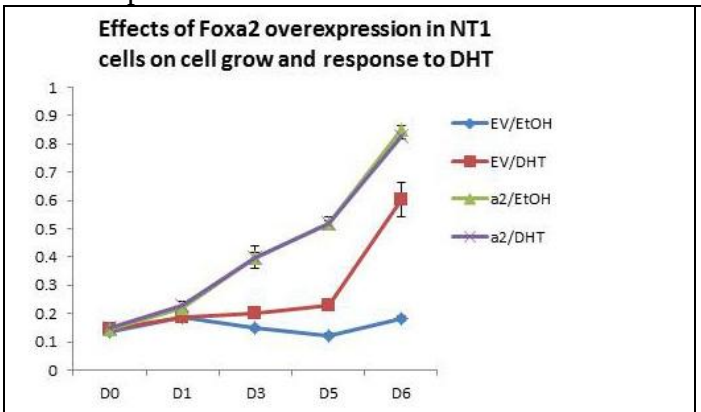


Figure 2. over-expression of Foxa2 enabled androgen independent prostate cell growth. NT1 cells with or without Foxa2 were cultured in 5% strip medium in the presence or absence of androgen. Cell growth curve was measured by WTS assays. Over-expression of Foxa2 increased NT1 cell growth even in the absence of androgen.

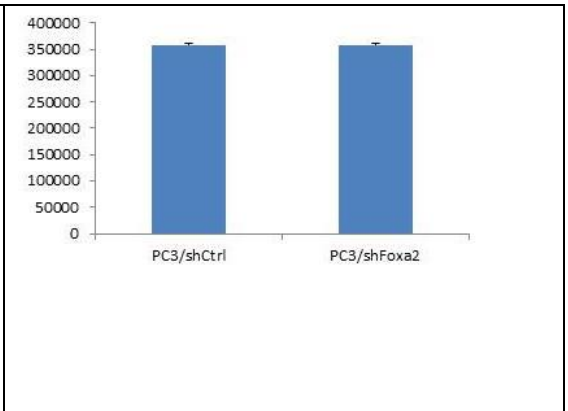


Figure 3. knocking-down Foxa2 did not affect PC3 cell growth in vitro. PC3 cells with or without shFoxa2 were seeded and grew for 3 days. Cell number was counted and compared between control and Foxa2 knocking-down cells.

E, We conducted western-blot and RT-PCR to examine the expression levels of AR and Foxa2 target genes. We found Foxa2 expression in NeoTag1 cells decreased AR level; however, AR

activity did not change. Foxa2 target genes were up-regulated in NT1/Foxa2 over-expressing cells.

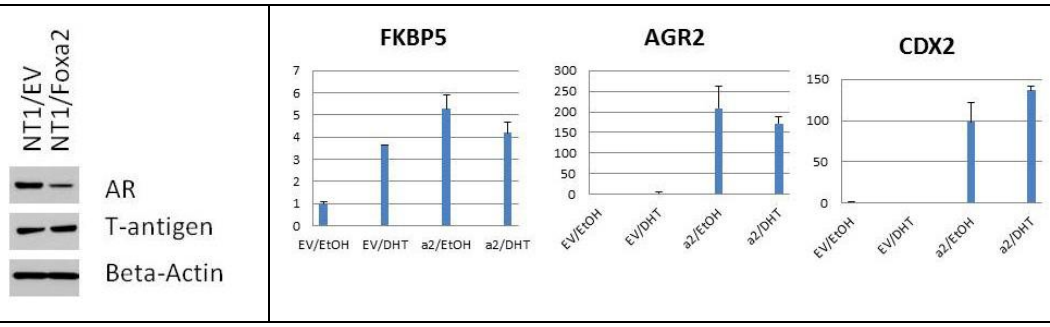


Figure 4: over-expression of Foxa2 reduced AR protein level, but not AR activity. Left panels: western blot of AR, SV40-T antigen and beta-actin. Although AR level is decreased, SV40 T-antigen, which is under the regulation of AR, did not change, indicating that AR trans-activity did not change. Right panels: FKBP5, an AR responsive gene was expressed in the absence of androgen, indicating that AR pathway in Foxa2 over-expressing NT1 cells is still active in the absence of androgen. AGR2 and CDX2, two Foxa2 target genes, were up-regulated in Foxa2 over-expressing NT1 cells. Research to study how Foxa2 regulated these genes is ongoing now.

Task 2. To determine if the expression of Foxa2 facilitates castration resistant prostate cancer growth in the bone.

2c. To determine if knocking down Foxa2 in prostate cancer cells impairs their growth in the bone.

PC3 cells (with or without Foxa2) was injected into tibias of nude mice (both nude and SCID mice are immune-deficient. The reason we chose nude instead of SCID mice is that androgen level in SCID mice is so low that prostate cancer cells won't be able to grow well in SCID mice), tumor growth in the tibias was monitored by weekly X-ray imaging. At end of the experiments, host mice were sacrificed and the tumor growth in tibias were analyzed by X-ray and micro CT scanning. Tissue samples were also processed for histological analysis. Our results showed that while control PC3 cells caused severe bone lytic lesions, PC3 cells with decreased Foxa2 levels generated significantly less bone lesions (figure 5).

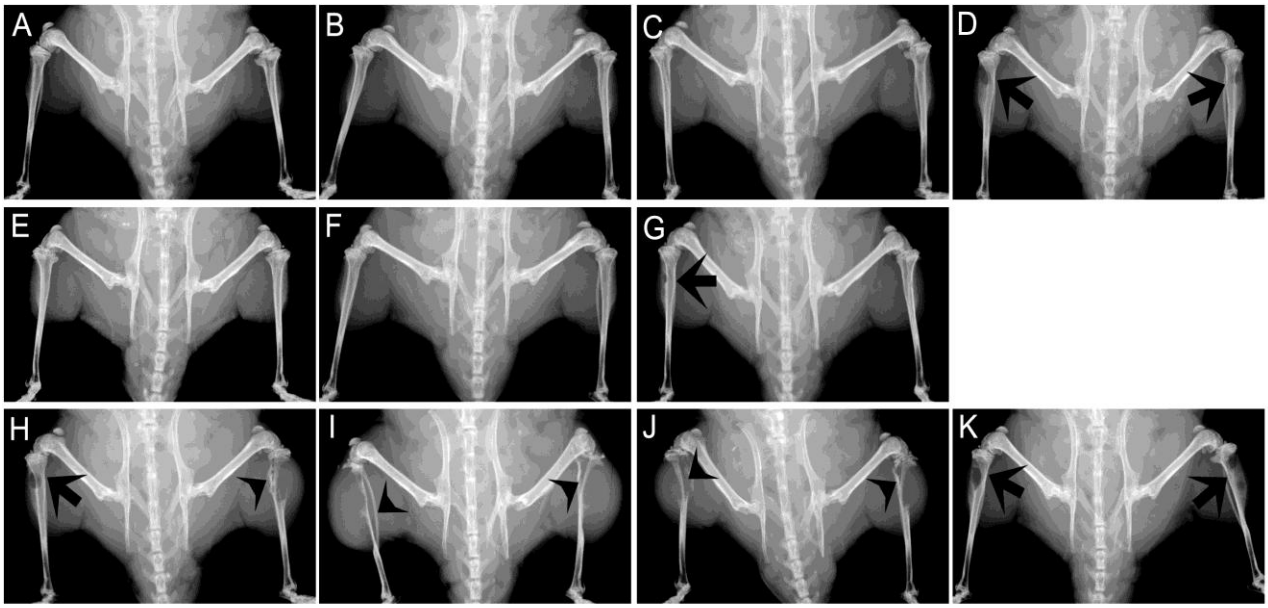


Figure 5. PC3 growth in the bones. A-G: PC3/Foxa2 knock-down cells; H-K: PC3/empty vector control cells. While control PC3 cells caused severe bone lytic lesions, PC3 cells with decreased Foxa2 levels generated significantly less bone lesions.

Task 3. To determine the suitability of pharmacological inhibition of Wnt-Foxa2-CXCR4 axis in conjunction with hormone deprivation to inhibit prostate cancer growth and castration resistant relapse in the bone and to prepare manuscript for publication.

We injected 22Rv1, a prostate cancer cell line,[5] into tibias of nude mice. Tumor growth in the tibias was monitored by weekly X-ray imaging. At end of the experiments, host mice were sacrificed and the tumor growth in tibias was analyzed by X-ray and micro CT scanning. Our results showed that 22Rv1 formed mixed osteolytic and osteoblastic lesions in the bone. The 22Rv1 can be a good model system to be used in aim 3.

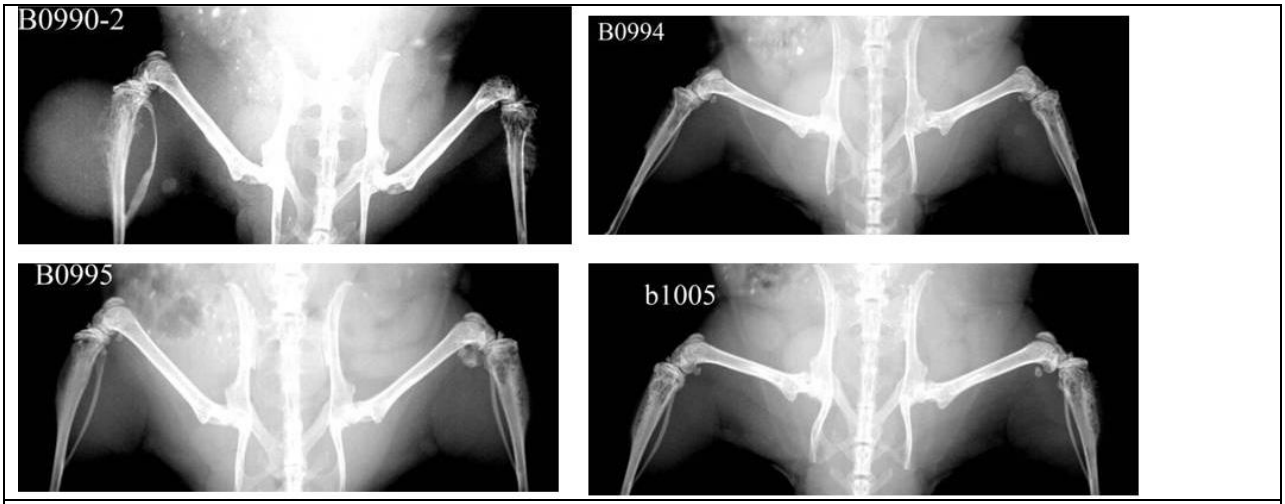


Figure 6. X-ray images of 22Rv1 bearing tibias.

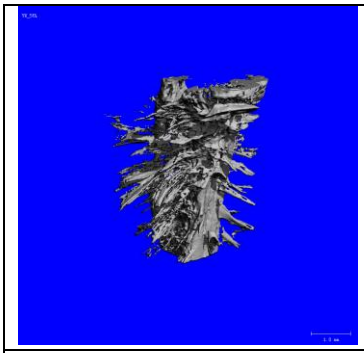
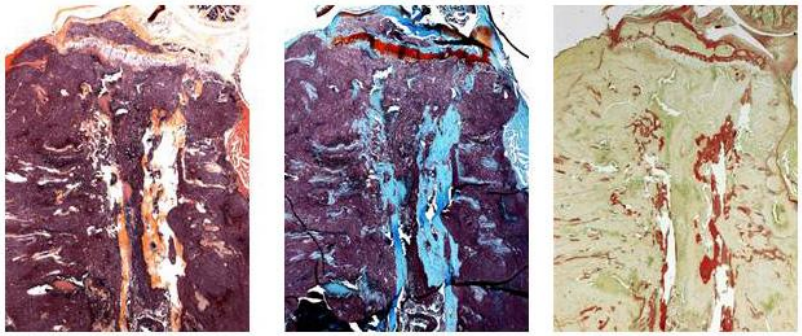
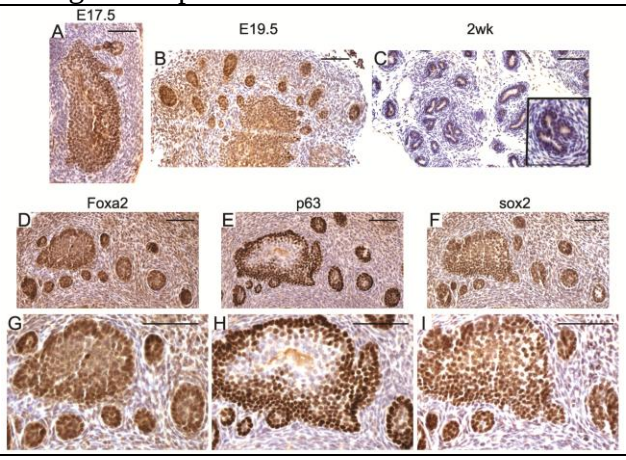
	

Figure 7. 22Rv1 cells caused mixed lesions in the bone.	Figure 8: histological staining of tibias bearing 22Rv1 cells. 22Rv1 cells formed mixed osteblastic and osteolytic lesions in the bone. Orange in the left, blue in the middle, and red in the right stainings show the newly formed bone outgrowing into surrounding muscle tissues.

Additionally, we have also examined the expression of Sox2, a Wnt/beta-catenin downstream target gene, during prostate development. We found Sox2 was expressed in embryonic prostate during development.

	Figure 8. Sox2 is expressed in budding prostate during embryonic development. Sox2, a wnt/beta-catenin target gene, is expressed in prostate buds during embryonic development. Foxa2 and p63, two known genes expressed during prostate morphogenesis were used as positive controls.

key research accomplishments

- Found that conditioned medium collected from CAFs can activate Wnt/beta-Catenin signaling in epithelial cells.
- Established Foxa2 over-expressing NeoTag1 cells, which will be used in later experiments in aim 2.
- Found that Foxa2 over-expression empowers androgen independent cell growth.
- Established Foxa2 knock-down PC3 cells, and found that knocking down Foxa2 in PC3 cells caused a decrease of CXCR4 level.
- Found that knocking down Foxa2 impairs PC3 cells mediated bone destruction.
- Identified 22Rv1 a good model system to be used in aim 3
- Examined the expression of Sox2 during prostate development.

Reportable outcomes
None.

Conclusions
Wnt/beta-catenin is implicated in prostate stromal/epithelial interaction. Foxa2 play an important role in the development of androgen independent prostate cancer and in prostate cancer mediated bone destruction.

References

1. Grossmann ME, Huang H, Tindall DJ. Androgen receptor signaling in androgen-refractory prostate cancer. *JNatlCancer Inst* 2001; **93**(22): 1687-97.
2. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *NatRevMolCell Biol* 2003; **4**(12): 915-25.
3. Yu X, Wang YQ, Jiang M, et al. Activated beta-catenin in mouse prostate causes HGPIN and continuous prostate growth after castration. *The Prostate* 2009; **69**(3): 249-62.
4. Yu X, Wang Y, DeGraff DJ, Wills ML, Matusik RJ. Wnt/beta-Catenin activation promotes prostate tumor progression in a mouse model. *Oncogene* 2011; **30**(16): 1868-79.
5. Henry MD, Silva MD, Wen S, et al. Spiculated periosteal response induced by intraosseous injection of 22Rv1 prostate cancer cells resembles subset of bone metastases in prostate cancer patients. *Prostate* 2005; **65**(4): 347-54.

Appendice
None.